
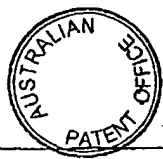


OPI DATE 03/08/98 APPLN. ID 62084/98
AOJP DATE 24/09/98 PCT NUMBER PCT/EP98/00103



AU9862084

(51) Internationale Patentklassifikation 6: C12N 15/10, C07H 1/08, B01D 21/26		A2	(11) Internationale Veröffentlichungsnummer: WO 98/30685
			(43) Internationales Veröffentlichungsdatum: 16. Juli 1998 (16.07.98)
(21) Internationales Aktenzeichen: PCT/EP98/00103		(81) Bestimmungsstaaten: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO Patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) Internationales Anmeldedatum: 9. Januar 1998 (09.01.98)			
(30) Prioritätsdaten: 97100331.4 10. Januar 1997 (10.01.97) EP (34) Länder für die regionale oder internationale Anmeldung eingereicht worden ist: DE usw.			
(71) Anmelder (für alle Bestimmungsstaaten ausser US): ROCHE DIAGNOSTICS GMBH BOEHRINGER MANNHEIM GMBH (DE/DE): Sandhofer Strasse 412-132, D-68305 Mannheim (DE) 116, D-68305 Mannheim, Germany		<p>Veröffentlicht Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.</p> 	
(72) Erfinder; und			
(75) Erfinder/Anmelder (nur für US): KUHNE, Wolfgang (DE/DE); Wolfbauerweg 10, D-82377 Penzberg (DE).			
(74) Anwälte: WEICKMANN, H. usw.; Kopcmikusstrasse 9, D-81679 München (DE).			
(54) Title: PURIFICATION AND/OR CONCENTRATION OF DNA BY CROSS-FLOW FILTRATION, SEPARATION OF ENDO-TOXINS FROM A NUCLEIC ACID PREPARATION			
(54) Bezeichnung: REINIGUNG ODER/UND KONZENTRIERUNG VON DNA DURCH CROSS-FLOW-FILTRATION, ABTRENNUNG VON ENDOTOXINEN AUS EINER NUCLEINSÄURE-PRÄPARATION			
(57) Abstract			
<p>The invention concerns a method of purifying and/or concentrating nucleic acids in a solution, the solution containing nucleic acid being guided tangentially past one or a plurality of semipermeable membranes, such that the nucleic acid molecules are retained by the membranes and substances having a lower molecular weight can pass through the membranes and/or be adsorbed thereat, so that a purified and/or concentrated nucleic acid solution is obtained. The same method is carried out to separate endotoxins from a nucleic acid preparation. The invention further concerns the use of a cross-flow filtration system for purifying and/or concentrating nucleic acids in a solution and for separating endotoxins from a nucleic acid preparation. The invention also concerns the use of the nucleic acids purified and/or concentrated by the cross-flow filtration system for cloning, transformation, transfection and microinjection into cells, for use in gene therapy processes, DNA vaccination and/or for polymerase chain reaction (PCR).</p>			
(57) Zusammenfassung			
<p>Die Erfindung betrifft ein Verfahren zur Reinigung oder/und zur Konzentrierung von Nucleinsäuren in einer Lösung, wobei man die Nucleinsäure enthaltende Lösung tangential an einer oder mehreren semipermeablen Membranen vorbeileitet, so daß die Nucleinsäure-Moleküle von den Membranen zurückgehalten werden und Substanzen mit einem geringeren Molekulargewicht durch die Membranen durchtreten können oder/und an der Membran adsorbiert werden und man eine gereinigte oder/und konzentrierte Nucleinsäure-Lösung erhält. In derselben Weise wird zur Abtrennung von Endotoxinen aus einer Nucleinsäure-Präparation verfahren. Weiter betrifft die Erfindung die Verwendung einer Cross-Flow-Filtrationsanlage zur Reinigung oder/und zur Konzentrierung von Nucleinsäuren in einer Lösung sowie zur Abtrennung von Endotoxinen aus einer Nucleinsäure-Präparation. Ferner die Verwendung der mit der Cross-Flow-Filtration gereinigten oder/und konzentrierten Nucleinsäuren zum Klonieren, zur Transformation, zur Transfektion, zur Mikroinjektion in Zellen, zur Verwendung bei Verfahren der Genterapie, der DNA-Vakzinierung oder/und zur Polymerase-Ketten-Reaktion (PCR).</p>			



BEST AVAILABLE COPY

Abstract

The invention concerns a method for purifying or/and concentrating nucleic acids in a solution, the solution containing nucleic acid being guided tangentially past over one or several semipermeable membranes such that the nucleic acid molecules are retained by the membranes and substances with a lower molecular weight can pass through the membranes or/and are adsorbed to the membrane to obtain a purified and/or concentrated nucleic acid solution. The same method is carried out to separate endotoxins from a nucleic acid preparation. In addition the invention concerns the use of a cross-flow filtration system to purify or/and concentrate nucleic acids in a solution and to separate endotoxins from a nucleic acid preparation. The invention also concerns the use of the nucleic acids purified and/or concentrated by cross-flow filtration for cloning, for transformation, for transfection, for microinjection into cells, for use in gene therapy methods, DNA vaccination or/and for the polymerase chain reaction (PCR).

PURIFICATION OR/AND CONCENTRATION OF DNA BY CROSS-FLOW
FILTRATION

Description

The present invention concerns a method for purifying or/and concentrating nucleic acids in a solution.

Nucleic acid purification methods are common methods in the field of molecular biology. In methods known from the prior art the isolated biological material, such as *E. coli* bacterial cells is for example centrifuged after they have been lysed (usually lysis with lysozyme or ultrasound) and the supernatant is shaken out with phenol. Subsequently an ultracentrifugation is carried out on a caesium chloride gradient (Birnboim & Doly, Nucl. Acid Res. 7 (1979) 1513-1523; Garger et al., Biochem. Biophys. Res. Comm. 117 (1983) 835-842).



for the toxic effect of endotoxins.

Another method for purifying nucleic acids is described in the QIAGEN® Plasmid Handbook (Qiagen Inc., Chatsworth, USA) and in EP-B 0 268 946. According to this the cell lysate obtained by the usual lysis is chromatographed on a QIAGEN® TIP which contains QIAGEN® resin (a support material based on silica gel). A disadvantage of this method is that DNA binding proteins are not completely detached from the DNA so that the plasmid preparation that is obtained contains a considerable amount of proteins and in particular endotoxins (e.g. from the membrane of the E. coli cell).

In another nucleic acid purification method after alkaline lysis of the biological material for example E. coli cells the centrifugation supernatant is chromatographed according to Birnboim & Doly under high salt conditions over anion exchange columns (e.g. Mono-Q, Source-Q from Pharmacia, Macrorep-Q from Biorad, Poros-Q from Perspective Biosystems or HyperD-Q from Biosepra, cf. Chandra et al., *Analyt. Biochem.* 203 (1992) 169-172; Dion et al., *J.Chrom.* 535 (1990) 127-147). Even after this purification step the plasmid preparation still contains impurities such as proteins and especially a considerable amount of endotoxins.

In yet another method for purifying nucleic acids a chromatography is carried out by gel filtration after alkaline lysis and subsequent phenol-chloroform extraction (McClung & Gonzales, *Anal.Biochem.* 177 (1989) 378-382; Raymond et al., *Analyt.Biochem.* 173 (1988) 124-133). This purification method is also not able to completely remove the impurities from the plasmid

preparation.

The said purification methods all have a final desalting and concentration step. This usually involves an isopropanol/ethanol precipitation of the nucleic acid with subsequent centrifugation and resuspension of the nucleic acid pellet in buffer (cf. e.g. Sambrook J. et al. (1989), Molecular Cloning; A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press). In this process a DNA solution is for example admixed with 1/10 volumes 4 M LiCl and subsequent with 0.7 volumes isopropanol at room temperature. Subsequently the precipitate that forms of the nucleic acid is centrifuged and the supernatant is discarded. The pellet which contains the nucleic acid is taken up in 70 % ethanol in a subsequent step, centrifuged again, the supernatant is discarded and, after drying the pellet, it is resuspended in a desired buffer. However, the isopropanol/ethanol precipitation method is only practical for applications in a laboratory where relatively small volumes are used.

Apart from the limitation to small volumes, the described isopropanol/ethanol precipitation method has other serious disadvantages. Thus for reasons of operational safety and environmental protection it is very unfavourable to use the required isopropanol/ethanol volumes on an industrial process scale.



A method for isolating and purifying nucleic acids for use in gene therapy is described in WO 95/21177 in which the purification is essentially by centrifugation, filtration, an affinity chromatography or a chromatography on an inorganic chromatographic material and a chromatography on an ion exchanger. In order to remove endotoxins the nucleic acid is treated with an endotoxin removal buffer which contains 10 % Triton® X100 and 40 mmol/l MOPS buffer (3-morpholino-1-propane sulfonate). A disadvantage of this method is that the nucleic acid purified in this manner is contaminated with the pharmacologically unsafe substances Triton® and MOPS. In addition, although it is possible to deplete endotoxins to a content of ca. 100 EU/mg DNA (QIAGEN News 1-96, 3-5), a more extensive removal of endotoxins is not possible. However, nucleic acid preparations with an even higher purity are required for a therapeutic application, like for example a gene therapy, which are as free as possible of all impurities (in particular substantially free of endotoxins). Above all the endotoxin content of plasmid DNA preparations has been a hitherto unsolved problem as for example described by Cotten et al., Gene Therapy 1 (1994) 239 - 246.

K.-G. Wahlund and A. Litzén (Journal of Chromatography, 461 (1989), 73-87; 476 (1989), 413-421) describe a method named "field flow fractionation (FFF)" suitable for analytical and micropreparative applications for separating protein mixtures and plasmids according to their respective molecular weights. As in a cross-flow filtration the approach flow on the ultrafiltration membrane is tangential but, in contrast to cross-flow filtration, the separation is based on the different migration of the molecules in the stream of carrier fluid. Hence the elution of the molecules to be

separated depends on their molecular size and the correlating diffusion coefficients. The separation is not continuous i.e. the molecules to be separated flow over the membrane only once during the separation process.

F.M. Fernandez, J.M. Romano and M.A. Otero (Acta Biotechnol. 12 (1992) 1, 49-56) describe the concentration of RNA in solution in a cross-flow filtration method with hollow fibre membranes. However, the purification of DNA or plasmid DNA is neither described nor made obvious.

G.W. Rembhotkar and G.S. Khatri (Analytical Biochemistry, 176, 337-374 (1989)) describe the purification of a λ phage lysate by means of tangential flow filtration. A subsequent λ phage DNA preparation is carried out with common methods using chloroform, phenol-chloroform treatment and ethanol precipitation.

A method for the isolation and purification of plasmid DNA from microorganisms is described in WO 96/36706 and 96/02658 A1 which were produced on a large scale. The cells are lysed by adding a lysis solution and heating to 70°C to 100°C in a flow heat exchanger and subsequently a clear supernatant is obtained by batch-wise centrifugation and diafiltration. The diafiltration is carried out in the dead-end modus but not by tangential overflow of the membrane according to a cross-flow fraction method. Afterwards a further purification is carried out by anion exchange chromatography and reversed phase HPLC. In this method only the cell lysis is carried out in a continuous process step, the further purification of the plasmid DNA is carried out in a batch

manner in common centrifuges and diafiltration devices.

In EP-A 96 101 628.4 the purification of nucleic acid preparations by an anion exchanger using a high salt gradient is suggested in order to obtain nucleic acid solutions which have a protein content of less than 0.1%, are free of impurities such as ethidium bromide, phenol, caesium chloride and detergents.

Hence in the prior art methods are not known which would enable large amounts of nucleic acids to be purified or concentrated.

10

The present invention concerns a method for purifying and/or concentrating plasmid DNA in solution wherein the solution containing the plasmid DNA is guided tangentially past one or several semi-permeable membranes such that the plasmid DNA molecules are retained by the membranes and substances with a

15 lower molecular weight can pass through

20

25



the membranes to obtain a purified or/and concentrated plasmid DNA solution.

It has now been found that nucleic acid solutions can be purified and concentrated with the method of the present invention using a cross-flow filtration system. In this connection a surprising and new feature is that the nucleic acids are not damaged by the cross-flow filtration (CFF). Previously it has always been assumed that the shear forces occurring in the CFF would lead to damage of nucleic acids in particular to strand breaks. Therefore CFF was previously only used to concentrate and diafiltrate proteins. In addition the method of the invention not only enables nucleic acids to be obtained in large amounts and of a desired purity, but the method of the present invention also avoids the use of organic solvents which is a major advantage toxicologically as well as with regard to safety and environmental aspects.



The method of the present invention is used to purify or/and concentrate linear or circular nucleic acids, preferably plasmid DNA and most preferably circular plasmid DNA. In this connection the size of the nucleic acid is preferably in the range of ≥ 150 base pairs, particularly preferably in the range of 1 kbp - 200 kbp. In the method according to the invention the nucleic acid can be purified or/and concentrated batch-wise but the method is preferably carried out continuously. Any volume size can be processed but it is preferable to process a solution with a volume of 1 to 10,000 l, particularly preferably of 1 - 100 l. The solution containing the nucleic acids is guided past the membrane or membranes under suitable pressure conditions whereby the cross-flow pressure is preferably larger than the transmembrane pressure. It is particularly preferable to operate at a transmembrane pressure of 0.2 to 3.0 bar and most preferably of 0.8 - 1.5 bar in which case the cross-flow pressure is larger than the transmembrane pressure. The retentate flow rate (RF) can be varied over a wide range and it is preferably to operate with an RF of 100 l/h·m² to 4,000 l/h·m². The process can also be carried out at varying temperatures and it is preferable to work in a temperature range of 4°C - 25°C.

In order to separate the solution containing nucleic acids from low molecular impurities and in particular from endotoxins, common membranes are used such as



membranes made of polyether sulfone (PES), modified PES, polyvinylidene difluoride (PVDF), cellulose triacetate or regenerated cellulose. Hollow fibre coil modules are also suitable for the method according to the invention. Membranes with an exclusion size of 1 - 1000 kilodalton (kD) are preferably used, 10 - 300 kD is more preferred and 10 - 100 kD is most preferred. The endotoxin depletion factor (ratio of endotoxin content of the nucleic acid preparation before cross-flow filtration to the endotoxin content of the nucleic acid solution after cross-flow filtration) that is achieved in the present invention is at least 10 : 1, preferably at least 200 : 1. The endotoxin content of the solution is very low after cross-flow filtration and is preferably < 0.1 EU/mg nucleic acid. The nucleic acids obtained in the present invention are essentially undamaged and essentially have no single-strand or double-strand breaks.

In particular a plasmid DNA purified according to the invention exhibits only one dominant band after gel electrophoretic separation which corresponds to the "covalently closed circle" conformation. Furthermore, apart from small amounts of the open circle and linearized circle conformations, no other bands are present.



Examples

In the described experiments membranes of the OMEGA type made of modified polyether sulfone from the Filtron Company (order No. 6S 100C01 exclusion size 100 kD), membranes made of cellulose acetate from the Sartorius Company or PVDF membranes from the Millipore Company were used. A membrane with an exclusion limit of 100 kilodalton was used in particular for endotoxin separation. In order to check the separation of endotoxins, E-toxate® from the Sigma Company (order No. 210) was used as a spiking solution. The endotoxins were tested by the solid gel method in which the addition of a solution containing endotoxin to a limulus amoebocyte lysate solution (LAL solution) leads to a gel formation of the mixture. The gel formation is due to a coagulation cascade that occurs in several steps.

1. Cross-flow filtration (CFF) of a plasmid DNA solution

In order to examine CFF as a method for concentrating plasmid DNA, a production preparation of 2000 g E. coli biomass is lysed by alkali lysis, processed by Q-Sepharose and hydroxylapatite chromatography and the plasmid DNA solution that is obtained is used as a starting solution in the CFF.



1.1.1 Production of a starting solution

2000 g wet *E. coli* biomass from the fermenter is filled into depyrogenized centrifuge beakers. 22.5 l resuspension buffer (50 mmol/l Tris-HCl, 10 mmol/l EDTA- Na_2 , pH 8 ± 0.2) is added and slowly stirred (ca. 35 rpm) for at least 24 hours at $5 \pm 4^\circ\text{C}$ until the biomass is completely suspended. In this process the temperature of the suspension is slowly increased to 25°C .

22.5 l 0.2 mol/l NaOH, 1 % SDS is added to the suspension while stirring at ca. 80 rpm and incubated for 5 minutes at 25°C . 22.5 l potassium acetate buffer (3 mol/l potassium acetate buffer pH 5.5) is added while stirring and the temperature of the biomass is reduced as rapidly as possible to 4°C . The biomass is centrifuged for 30 minutes at $26,000 \times g$ and 4°C . The supernatant which contains the plasmid DNA is isolated and filtered clear over a $5 \mu\text{m}$ candle filter.

In the next step a chromatography on Q-Sepharose is carried out. The decanted centrifuge supernatant is adjusted to a conductivity of 49 - 50 mS/cm by addition of TE buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA pH 8.5 ± 0.2) and cooled to $5 \pm 4^\circ\text{C}$. The entire chromatography is carried out at this temperature. The centrifugation supernatant is absorbed to the equilibrated column. Subsequently the column is washed with ca. 8 CV 10 mmol/l Tris-HCl, 1 mmol/l EDTA, 0.65 mol/l NaCl pH 8.5 ± 0.2 .

For the elution a gradient (5 CV buffer A (10 mmol/l Tris-HCl, 1 mmol/l EDTA, 0.65 mmol/l NaCl, pH 8.0 ± 0.2), 5 CV buffer B (10 mmol/l Tris-HCl, 1 mmol/l EDTA, 0.85 mol/l NaCl pH 8.0 ± 0.2)) is applied to the column

and the eluate is fractionated at a flow rate of 5 to 8 CV/h, the detection is carried out at 254 nm. The prepeak (impurities) is separated from the main peak (plasmid DNA) by collecting the main peak in a separate vessel starting from the ascending flank.

Subsequently a chromatography on hydroxylapatite (HA ceramic) is carried out at $5 \pm 4^\circ\text{C}$.

Equilibration buffer: 0.1 mol/l potassium phosphate, 6 mol/l urea pH 7.0 ± 0.2 .

Wash buffer 1: 0.15 mol/l potassium phosphate, 6 mol/l urea pH 7.0 ± 0.2 .

Wash buffer 2: 0.02 mol/l potassium phosphate buffer pH 7.0 ± 0.2 .

Elution buffer: 0.5 mol/l potassium phosphate pH 7.0 ± 0.2 .

The detection is carried out at 254 nm using a UV detector/recorder unit. A 1 % product solution (plasmid DNA) is used as a calibration solution that was measured with a calibrated photometer.

The Q-Sepharose pool is adjusted to a final concentration of 1.1 mmol/l calcium chloride and absorbed onto the equilibrated column.

Then the column is successively washed at a flow rate of 5-8 CV/h with:

diluted to 1000 ml with TE buffer and then again concentrated with the aid of CFF to its initial volume of 100 ml (concentrate 1). The dilution and concentration step is repeated four times in succession. After each concentration step a sample is taken from each concentrate (samples: concentrate 2, 3, 4, 5) and the endotoxin concentration of the sample is analysed with the limulus-amoebocyte lysate method..

3. Results

3.1 CFF of the plasmid DNA solution

The plasmid DNA solution can be concentrated and diafiltered without difficulty using the CFF. The results of the examination are summarized in the following table.

Parameter	PLASMID DNA initial solution (HA pool)	PLASMID DNA after CFF	Diafiltration buffer (TE): 10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 8.0
Volume (ml)	3750	505	-
OD _{260/280}	1.89	1.90	-
Conductivity (mS/cm)	26.5	1.11	1.11
pH	6.99	7.93	7.98
Yield (mg)	763	666	-

An aliquot of the plasmid DNA after completion of the CFF is applied at various concentrations to an agarose gel. The agarose gel that is shown shows the DNA length standard No. II (fragment sizes: 125, 564, 2027, 2322, 4361, 6557, 9416, 23130 bp) in lanes 1 and 10 and the DNA length standard No. III (fragment sizes: 125, 564,

831, 647, 1375, 1584, 1904, 2027, 3530, 4268, 4973, 5148, 21226 bp) in lanes 2 and 9. pBR322 (4162 bp) is applied as a reference plasmid in lane 3 which was purified by a conventional caesium chloride gradient method. It is known that plasmid DNA purified by this method essentially contains plasmid DNA which corresponds to the covalently closed circle conformation (dominant supercoiled band). The plasmid DNA (pCMV-CAT) purified by the method according to the invention is applied in different amounts in lanes 4, 5 and 6. The plasmid DNA purified according to the invention, like the reference plasmid DNA (lane 3), essentially shows a dominant band. This plasmid DNA band corresponds to the covalently closed circle conformation (dominant supercoiled band). This shows that the plasmid DNA isolated according to the invention is not damaged and retains its original conformation. This therefore rules out the possibility that the plasmid DNA is fragmented during the CFF or is converted into an undesired plasmid DNA conformation.

Legend:

1% agarose gel

- Lane 1: DNA length standard II (Boehringer Mannheim GmbH; Cat. No. 236250)
 - Lane 2: DNA length standard III (Boehringer Mannheim GmbH, Cat. No. 528552).
 - Lane 3: pBR322 (Boehringer Mannheim GmbH, Cat. No. 481238) (0.4 μ g)
 - Lane 4: pCMV-CAT after CFF, 0.19 μ g (bulk active substance solution)
 - Lane 5: pCMV-CAT after CFF, 0.45 μ g (bulk active substance solution)
 - Lane 6: pCMV-CAT after CFF, 0.71 μ g (bulk active substance solution)
 - Lane 7: TE buffer
 - Lane 8: pBR322 (Boehringer Mannheim GmbH, Cat. No. 481238) (0.4 μ g)
 - Lane 9: DNA length standard III (Boehringer Mannheim GmbH; Cat. No. 528552)
 - Lane 10: DNA length standard II (Boehringer Mannheim GmbH, Cat. No. 236250).
-

3.2 Endotoxin depletion by CFF

The following table shows that the endotoxins are already substantially removed after the first concentration step. The additional CFF reduces the endotoxin concentration down to the detection limit of the test method.

Sample	Retentate volume [ml]	Measured endotoxin concentration in the retentate [EU/ml]
Initial solution	100	6-12
Concentrate 1	100	0.06 - 0.60
Concentrate 2	100	0.06 - 0.60
Concentrate 3	100	0.06 - 0.60
Concentrate 4	100	0.06 - 0.60
Concentrate 5	100	< 0.06
Diafiltration buffer	-	< 0.06

The claims defining the invention are as follows:

1. Method for purifying or/and concentrating plasmid DNA in a solution,
wherein
the solution containing plasmid DNA is guided tangentially past one or several semi-permeable membranes in a process that proceeds continuously such that the plasmid DNA molecules are retained by the membranes and substances with a lower molecular weight can pass through the membranes to obtain a purified or/and concentrated plasmid DNA solution.
2. Method as claimed in claim 1,
wherein
the nucleic acid has a size of ≥ 150 base pairs.
3. Method as claimed in one of the previous claims,
wherein
a solution with a volume of 1 - 10,000 l is processed.
4. Method as claimed in one of the previous claims,
wherein
a solution with a volume of 1 - 100 l is processed.
5. Method as claimed in one of the previous claims,
wherein
the solution is guided past the membrane(s) under pressure whereby the cross-flow pressure is larger than the transmembrane pressure.



6. Method as claimed in claim 5,
wherein
a transmembrane pressure of 0.2 - 3.0 bar is used.
7. Method as claimed in one of the previous claims,
wherein
a retentate flow rate of 100 - 4000 l/h·m² is used.
8. Methods for purifying and/or concentrating plasmid DNA in a solution,
substantially as hereinbefore described with reference to the Examples.

DATED this 31st day of March, 2000

ROCHE DIAGNOSTICS GmbH

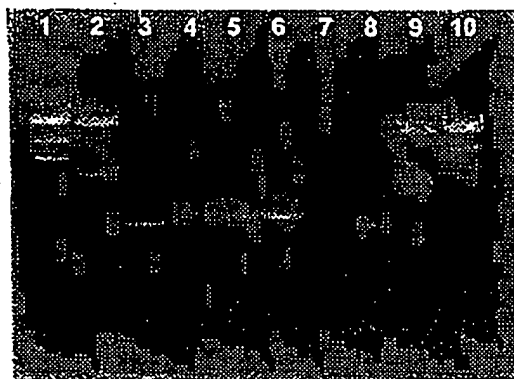
By its Patent Attorneys

DAVIES COLLISON CAVE



1/1

Figure 1



THIS PAGE BLANK (USPTO)

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)